

study was conducted to fractionate, tentatively identify and semi-quantitate the major proteins in the 3 SCW residual lipid fractions by sodiumdodecylsulfate polyacrylamide gel electrophoresis and densitometry. SCW, LDLF, MDLF, and HDLF contained 7, 3, 5, and 8 protein components, respectively. α -lactalbumin and β -lactoglobulin accounted for 44.6 to 80.8% of the total proteins in the SCW residual lipid fractions. Bovine serum albumin accounted for 4.4 to 8.5% of the total proteins in the 3 residual lipid fractions. HDLF contained the highest percentages of all protein components with molecular weights of ≥ 31 kilo Daltons, except xanthine oxidase.

KAMATH, S.R., MORR, C.V.: Identifizierung von Proteinen in Molkenrestlipidfraktionen von Schweizer Käse *Milchwissenschaft* 54 (3) 131–133 (1999).

54 Schweizer Käse (Molkenanalytik)

Es wurde gezeigt, daß Restlipide, die aus der Käsemolke nicht durch handelsübliches Trennverfahren entfernt sind,

die funktionellen Eigenschaften und Flavorstabilität von getrockneter Molke und Molkenproteinkonzentraten verschlechtern. Frühere Untersuchungen befaßten sich mit der Charakterisierung der Lipidbestandteile von 3 durch Hochgeschwindigkeits-Zentrifugierung von Schweizer Käse-Molke (SCW) hergestellter Restlipidfraktionen: Lipidfraktionen niedriger Dichte (LDLF), mittlerer Dichte (MDLF) und hoher Dichte (HDLF). In der vorliegenden Studie wurden die wesentlichen Proteine in den 3 SCW-Restlipidfraktionen durch Natriumdodecylsulfat-Polyacrylgelelektrophorese (PAGE) und Densitometrie fraktioniert, orientierend identifiziert und gemessen. SCW, LDLF, MDLF und HDLF enthielten 7 bzw. 3, 5 und 8 Proteinkomponenten. 44,6 bis 80,8% der Gesamtproteine in den SCW-Restlipidfraktionen bestanden aus α -Laktalbumin und β -Laktoglobulin. 4,4 bis 8,5% der Gesamtproteine in den 3 Restlipidfraktionen waren Rinderserumalbumin. HDLF enthielt die höchsten Anteile aller Proteinbestandteile mit Molekulargewichten von ≥ 31 kilo Daltons, mit Ausnahme der Xanthinoxidase.

Evaluation of commercial adjuncts for use in cheese ripening: 2. Ripening aspects and flavor development in cheese curd slurries prepared with adjunct lactobacilli

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1. Introduction

In a previous publication (13) it was shown that commercially available adjuncts vary considerably in their peptidase and esterase activities as well as their autolytic properties. Differences in the cell resistance to the autolytic process corresponded to differences in the rate of intracellular enzyme release. This work also revealed that little enzyme release was measured in cultures exhibiting high levels of intracellular enzyme activity with low levels of autolysis. The results from this work (13) led to the classification of cultures based on their levels of intracellular enzyme activities as well as their resistance to the autolytic process.

The commercial adjunct cultures of lactobacilli can be added to the *Lactococcus* starter to promote the ripening process in cheese (14, 20, 24, 26) and/or to eliminate bitterness (4, 8, 18). Attenuation of adjunct cultures by freeze shocking before addition to cheese milk can enhance the autolysis of adjunct cells without excess acid production during cheese-making process (11, 17). However, our understanding of specific contribution of adjunct cultures to flavor development during cheese ripening is limited (15).

Accelerated cheese ripening via cheese curd slurries was developed by KRISTOFFERSON *et al.* (22). The method led to a considerably rapid ripening because of the elevated temperature (32°C) used during the ripening process and the high moisture content of cheese slurry. Several researchers used the cheese slurry technique

as a reliable rapid tool for investigating the contribution of different agents to cheese ripening process (8, 14, 27, 28).

The purpose of the present work is to confirm buffer system results (13) in cheese slurry in order to study the influence of the cheese environment on the intracellular peptidase and esterase system of selected commercial adjunct cultures. The degree of enzyme release associated with the extent of cell lysis will also be determined. Results of this work should provide further needed information to select commercial cultures for their utilization as adjuncts in cheese-making trials.

2. Materials and methods

2.1 Culture selection, cell cultivation and freeze shocking

Among the 15 cultures described in our previous work (13) 3 groups of cultures were selected according to their peptidase and esterase activities and autolytic properties. *Lb. helveticus* I, and M and *Lb. casei* C, were high in peptidase activity, esterase activity and level of autolysis. *Lb. helveticus* L and *Lb. casei* A showed high peptidase and esterase activities with considerably low levels of autolysis. The third group composed of *Lb. helveticus* U and *Lb. casei* T displayed high autolysis with relatively low enzymatic activities. Cultures are arbitrarily identified with a letter to maintain confidentiality of commercial cultures.

For the cultivation of cells, MRS broth was inoculated with an active culture of the organisms and sub-cultured in the same medium. At early stationary phase, cells were harvested by centrifugation at 1500 g for 30 min at 4°C. The cell pellet was washed twice with 0.01 M potassium phosphate buffer, pH 7.0 and resuspended in the same buffer to obtain a total viable cell concentration of approximately 10^9 cfu/ml. The cell suspension was frozen at -20°C for 24 h and thawed in a water bath at 40°C prior to use.

2.2 Preparation of cheese curd for use in slurries

Fresh Cheddar cheese curd was made from pasteurized (76°C, 16 s) milk containing 3.2% fat in 40 l vat according to the conventional method of Cheddar cheese-making (19). A commercial starter culture of mesophilic *Lactococcus* strains (type DVS 850, Chr. Hansen's Lab*, Milwaukee, WI) was added (0.1%, w/v) to the milk at 30°C. Coagulant (Chymax Chr. Hansen Lab. Milwaukee, WI) was added to milk (0.02%, v/v) and the rennet-treated milk was left to coagulate under quiescent conditions. The coagulum was cut and cooked to 39°C over 30 min and held at this temperature for 15 min. After whey drainage, the curds were then cheddared and milled at pH 5.6. The unsalted curd was used to prepare the cheese slurries. Precautions were taken throughout manufacturing to avoid contaminant microorganisms.

2.3 Preparation of cheese slurries

Cheese slurries were prepared under controlled microbiological conditions following the procedure of FARKYE *et al.* (15). Cheese curd (85g), adjunct cell suspension (1.5 ml), NaCl (3 g) and sterile water (10 ml) were blended to a fine paste in a sterile Waring blender jar. The slurry was transferred aseptically into sterile culture tube, which was capped loosely and incubated at 32°C for 5 days under anaerobic conditions using a Gas-Pack 100 system (BBL, Cockeysville, MD). A control slurry was prepared without adding the cell suspension of adjunct lactobacilli.

2.4 Chemical and Microbiological analysis of cheese slurries

Duplicate samples of the cheese slurries were analyzed for moisture, fat, salt, total protein and pH (2). Proteolysis in cheese slurries was assessed by measuring the water-soluble nitrogen (WSN) (23) and the concentration of free amino groups by the cd-ninhydrin method (16). Urea polyacrylamide gel electrophoresis (PAGE) was also performed on cheese slurry samples using the method of ANDREWS (1). Lipolysis was monitored by analyzing the total free fatty acids according to the procedure of DEETH *et al.* (7). Lactobacilli count in ripened cheese slurries were determined by plating on MRS agar after incubation at 32°C for 48 h.

2.5 Assay of enzyme activities in cheese slurries

Lysine *p*-nitroanilides and *p*-nitrophenyl caproate were used to measure aminopeptidase and esterase activity in phosphate buffer extract of cheese slurry as described by EL ABOUDI *et al.* (10).

2.6 Organoleptic evaluation of cheese slurries

The cheese slurries were tested for aroma and taste by a laboratory panel of 5 people experienced in Cheddar cheese evaluation. Coded samples were evaluated for Cheddar flavor intensity, bitterness or other detectable flavor as strong, moderate and weak.

3. Results and discussion

3.1 Compositional and microbiological analysis

Both the control and adjunct-treated slurries had similar compositional analysis with mean and standard deviation of $52.45 \pm 1.18\%$ moisture, $24.80 \pm 1.04\%$ fat, $18.70 \pm 0.52\%$ protein and $3.83 \pm 0.30\%$ salt/moisture. The experimental and control slurries displayed a similar pH (5.3 ± 0.16). This indicates no substantial acid production by added freeze-shocked lactobacilli. Average initial viable cell counts on MRS media of cheeses with added adjunct lactobacilli and without added adjunct lactobacilli were approximately 10^7 cfu/g and $\leq 10^2$ cfu/g, respectively.

3.2 Enzyme activity in cheese slurries

The results in Fig. 1 revealed that the ripened cheese slurries with adjunct lactobacilli exhibited considerably higher aminopeptidase and esterase activity compared to the control. The levels of esterase and peptidase in the control slurries were rather low and showed little increase after 5 days of incubation at 32°C. These results may reflect very little autolysis and release of enzymes from the starter cultures used in the cheese-curd manufacturing process. On the other hand, slurries made with *Lb. helveticus* M and I, selected for their high enzymatic potentials and high autolytic properties, exhibited the highest levels of peptidase and esterase release. The presence of high enzyme activity due to the lysis of attenuated cells of lactobacilli in cheese matrix has been demonstrated in other reports (12, 17, 19). The slurries prepared with *Lb. casei* C showed unexpectedly low enzymatic activities which may be due to low stability of the esterase and peptidase systems of this culture in the cheese environment. These results suggest that at cheese slurry conditions, the aminopeptidase activity of some commercial adjuncts investigated may be reduced while other strains are not affected. These findings are in agreement with the numerous studies on the peptidases of lactic acid bacteria described in the literature (6, 18, 26). The slurries to which the cultures with low autolysis *Lb. casei* A and *Lb. helveticus* L were added, displayed little enzyme activity as expected. These results confirm our previous findings in buffer system (13) and indicate that cultures with high enzymatic activity may not have an impact during ripening of cheese if they are resistant to the autolytic process. Further some highly autolytic cultures may not impact cheese ripening because of the poor stability of their enzymes.

3.3 Ripening assessment in cheese slurries

Mean concentration of water-soluble N and cd-reactive amino groups (Table 1) are consistent with the preceding results of peptidase activity in cheese slurries (Fig. 1). A higher level of WSN was measured in most adjunct treated-cheese slurries compared to the

*Any mention of a company name or product name is for scientific clarity only

control slurry without adjunct. In fact, *Lb. helveticus* I, M and U exhibited the highest degree of protein breakdown in the cheese slurries due to their high peptidase activity release. The % WSN content of *Lb. helveticus* strain I, M and U were 55, 60 and 75% higher, respectively, than the control. *Lb. casei* A revealed little proteolysis. This strain, as well as *Lb. helveticus* L, are rather resistant to the autolytic process. Analysis of water soluble extract of the cheese slurry by the cd-ninhydrin method (which is highly sensitive for free amino acids) showed that the slurries prepared with most adjunct lactobacilli contained considerably higher concentration of free amino acids than control. The differences observed between the control slurry and adjunct-treated slurries appear to be attributed to the high activity of adjunct peptidases and to the greater ability of the adjunct cultures to release their intracellular peptidases in the cheese matrix (9, 19). The levels of WSN and FAA in the adjunct-treated slurries were almost comparable to those found in 3 month-old mild flavored Cheddar cheese. The higher concentration of FAA in adjunct-treated slurries suggests that the peptidase of lactobacilli would contribute significantly to liberation of amino acids during cheese maturation. BROOME *et al.* (5) found higher levels of soluble amino N and individual amino acids in Cheddar cheese curd inoculated with *Lb. casei* and attributed this increase to high peptidase activity in the cheese curd containing the adjunct cultures. Reported data on cheese slurry studied by FARKYE *et al.* (15) showed that adjunct lactobacilli were more proteolytic and produced amino acids more readily in adjunct-treated slurries than slurry with starter lactococci alone. Small peptides and free amino acids in WSN of Cheddar cheese are thought to be the major contributors to cheese flavor (3).

Table 1: Mean and standard deviation (SD±) of proteolysis and lipolysis indices in cheese slurries inoculated with different strains of adjunct lactobacilli

Cheese slurry	Proteolysis indices*		Lipolysis indices*
	WSN ^a % of TN	cd-free amino acids ^b (mM leucine equiv.)	total FFA (mmol equiv./g)
Control (fresh)	4.80±0.84	0.22±0.067	10.10±1.03
Control (5 d)	13.50±1.07	0.70±0.068	15.10±0.92
<i>Lb. helveticus</i> I	19.81±1.42	3.10±0.17	24.40±1.21
<i>Lb. helveticus</i> U	18.50±1.25	2.00±0.14	22.40±1.15
<i>Lb. helveticus</i> C	14.60±0.8	1.10±0.091	20.50±0.84
<i>Lb. helveticus</i> M	21.20±1.16	3.80±0.16	21.70±1.03
<i>Lb. casei</i> T	14.60±0.77	1.45±0.083	15.40±0.88
<i>Lb. helveticus</i> L	15.00±1.70	2.60±0.20	17.40±0.80
<i>Lb. helveticus</i> A	7.20±1.03	0.70±0.065	16.00±1.13
Mild flavored Cheddar (90 d)	16.00	2.30	15.00

* Results are means of duplicate determination on 2 replicates

^aWater-soluble nitrogen as % of total N

^bTotal free amino groups expressed as mM leucine equivalent

The results describing the rate of soluble nitrogen (WSN) and free amino acids formation in the cheese slurries are to some extent confirmed by the gel electrophoretograms of cheese slurries after ripening (Fig. 2). Adjunct-treated slurries showed more degradation of α_{s1} -casein, indicating greater peptidase activity in the curd compared to control slurry. The rate at which α_{s1} -casein was degraded appeared to be higher in cheese slurries inoculated with the highly autolytic and proteolytic

cultures *Lb. helveticus* I, M and U when compared to culture strains A and L. In fact, the 2 latter cultures showed degradation pattern almost identical to the control slurry. Cheese slurries prepared with most adjunct lactobacilli also had somewhat higher concentration of γ -caseins, indicating higher extent of β -casein degradation due to a relatively higher level of peptidase activity compared to the control slurry. It was of interest to notice that electrophoretic profile for casein degradation in cheese slurries with adjunct *Lb. helveticus* I and U was comparable to 3-month-mild flavour Cheddar cheese with good sensory evaluation. In several reports, addition of adjunct lactobacilli to cheese curd led to higher rate of proteolysis and more intense flavor development during cheese ripening (4, 9, 19). Moreover, LANE and FOX (24) reported that the addition of adjunct lactobacilli to starter free cheese acidified with glucono δ -lactone caused a significant increase in proteolysis. Proteolysis increase was less apparent when the lactobacilli were added to the cheese starter. This may be due to the lack of available substrate for the enzyme for both cultures. LYNCH *et al.* (25) also demonstrated that addition of lactobacilli led to higher levels of amino acids formation in the cheese as well as differences in the profiles of individual amino acids.

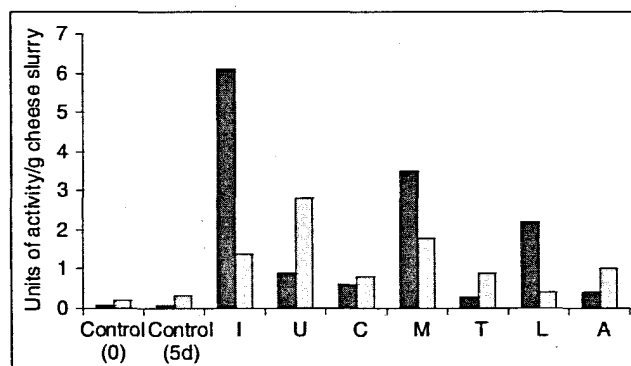


Fig. 1: Aminopeptidase and esterase activity in the cheese slurries inoculated with different strains of adjunct lactobacilli (*Lb. helveticus* I, U, M, L and *Lb. casei* C, T, A). ■ peptidase (Lys), ▨ esterase (C6)

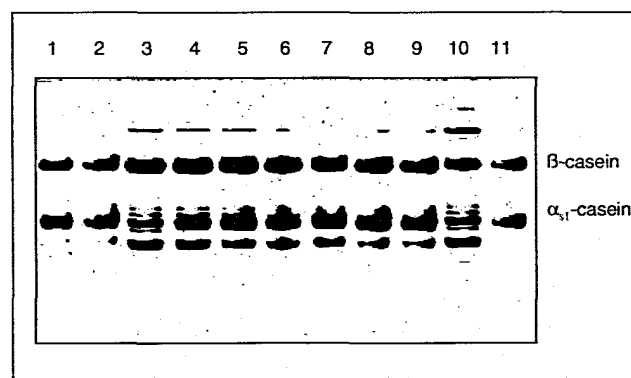


Fig. 2: Gel electrophoresis of cheese slurries inoculated with different strains of adjunct cultures and incubated for 5 d at 30 °C. 1: control 0 time (no adjunct), 2: control 5 d (no adjunct), 3–9: cheese slurries with adjunct strains *Lb. helveticus* I, U, *Lb. casei* C, *Lb. helveticus* M., *Lb. casei* T, *Lb. helveticus* L and *Lb. casei* A, respectively. 10: Cheddar cheese (3 month-old mild flavored), 11: Na-caseinate

The relative concentration of total free fatty acids (FFA) assessed in cheese slurries (Table 1) revealed higher extent of lipolysis in most adjunct treated-slurries. Greater level of FFA liberation occurred in slurries prepared with adjunct I, M, U and C compared to control slurry. The results show that the adjunct strains tested differed from each other in their ability to contribute to FFA formation. In fact, the stability and activity of esterase system of lactobacilli were reported to be strain specific (12, 20). The overall concentration of FFA in the adjunct-treated slurries was similar to the values found in 3 month-old mild flavored Cheddar cheese. In general, trends observed in the behavior of adjunct cultures for lipolysis in cheese slurries were similar to those observed for proteolysis. This indicates that adjunct cultures contribute to lipolysis in cheese and different adjunct strains have different lipolytic activity in cheese curd system.

3.4 Organoleptic evaluation

Cheese slurries were evaluated on the basis of flavor and aroma. All adjunct treated slurries graded best for cheese flavor and aroma. The control slurries tasted like acidified milk and lack the characteristic cheesy aroma, whereas, those slurries containing adjunct had definite cheese flavor aroma. The intensity of the flavor varied among different adjunct culture slurries. *Lb. helveticus* I treated slurry exhibited Cheddar cheese aroma with slight sulphuric character and less acidity while those made with adjunct M and U exhibited defined cheesy flavor with slight sweetness. These results demonstrate that adjunct lactobacilli can have a strong impact on the enhancement of cheese flavor development. Evaluation of adjunct treated-slurries in other studies (9, 28) also indicated the ability of adjunct lactobacilli especially *Lb. casei* and *Lb. helveticus* to produce cheese flavor and to improve sensory quality of cheese slurries. Several reports (8, 19, 24, 25) have demonstrated that addition of non starter lactic acid bacteria, to cheese curd considerably enhance flavor development more than starter alone.

4. Conclusion

The cheese slurries results obtained in this study approach closely the trends in the results obtained in buffer system study (13). Addition of freeze-shocked adjunct lactobacilli to cheese curd made with starter culture gave a clear indication of their potential contribution to proteolysis and lipolysis during cheese ripening. Ripening indices (WSN, FAA, PAGE and FFA) revealed that adjunct lactobacilli increase the extent of protein and fat degradation in model cheese slurry. Actually, addition of adjunct cultures with high autolytic properties and high enzyme activity such as *Lb. helveticus* strains I or M led to higher concentrations of FAA and FFA in cheese slurries compared to control slurry. The presence of high peptidase and esterase activity in extracts from slurries made with adjunct strains of lactobacilli with enhanced autolytic characteristics reflect the importance of cell lysis during cheese ripening. On incubation at 32 °C for 5 days, the slurries prepared with these adjunct strains underwent proteolytic and lipolytic changes resembling 3 month-old mild flavored Cheddar cheese. Moreover, a sensory panel determined that

cheese slurries containing the lactobacilli adjunct exhibited appreciable cheese aroma coincident with considerably high levels of proteolysis and lipolysis. On the other hand, little contribution to both proteolysis and lipolysis could be measured when adjunct culture with poor autolytic properties such as strain *Lb. casei* A was added to the slurry system. From these findings and previous work in buffer system (13) it is possible to select adjunct strains of lactobacilli for the stability of their enzymes as well as for their improved autolytic properties in the cheese environment. Some adjunct cultures can have positive effect in improving cheese flavor and aroma due to high intracellular enzyme activities. Further work is continuing on the use of the most promising attenuated adjunct cultures in actual cheese-making conditions of low- and full-fat Cheddar.

Acknowledgements

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6. Summary

MADKOR, S.A., EL-SODA, M., TONG, P.S.: **Evaluation of commercial adjuncts for use in cheese ripening: 2. Ripening aspects and flavor development in cheese curd slurries prepared with adjunct lactobacilli.** *Milchwissenschaft* **54** (3) 133–137 (1999).

56 Cheese ripening (adjunct lactobacilli)

Seven strains of freeze-shocked adjunct lactobacilli were evaluated for their proteolytic and lipolytic activity in cheese slurry system. The ripened cheese slurries with adjunct lactobacilli exhibited considerably higher aminopeptidase and esterase activity compared to the control after incubation for 5 d at 32 °C. Slurries made with *Lb. helveticus* I and M which were selected for their high enzymatic potentials and high autolytic properties exhibited the highest levels of peptidase and esterase activity. The slurries to which the poorly autolytic cultures *Lb. casei* A and *Lb. helveticus* L were added displayed very little enzyme activity. Higher levels of water soluble nitrogen (WSN) and total free amino acids (FAA) were measured in most adjunct treated-cheese slurries compared to the control slurry without adjunct. *Lb. helveticus* I, M and U exhibited the highest rates of protein breakdown in the cheese slurries as indicated by the high levels of WSN and FAA due to their high peptidase activity release and low resistance to autolytic process. *Lb. casei* A and *Lb. helveticus* L which were rather resistant to the autolytic process showed little proteolysis after 5 d of ripening. Adjunct-treated slurries showed more degradation of proteins on polyacrylamide

gel electrophoresis (PAGE) indicating greater peptidase activity in the curd compared to control slurry. The relative concentration of total free fatty acids (FFA) assessed in cheese slurries revealed high extent of lipolysis in most adjunct treated-slurries. Greater level of FFA liberation occurred in slurries prepared with adjunct I, M, U and C compared to control slurry. A sensory panel revealed that cheese slurries containing the lactobacilli graded best for cheese flavor and aroma but the intensity of the flavor varied among different adjunct culture slurries.

MADKOR, S.A., EL-SODA, M., TONG, P.S.: **Bewertung handelsüblicher Zusätze bei der Käsureifung. 2. Reifungsaspekte und Flavourentwicklung in Käsebruchaufschwemmungen mit Laktobazillen-Zusatz.** *Milchwissenschaft* **54** (3) 133–137 (1999).

56 Käsureifung (Laktobazillenzusatz)

7 Stämme schockgefrorener Laktobazillenzusätze wurden auf ihre proteolytische und lipolytische Aktivität im Käsebruch-Aufschwemmungssystem bewertet. Die gereiften Käsebruch-Aufschwemmungen mit Laktobazillenzusatz wiesen wesentlich höhere Aminopeptidase- und Esteraseaktivität im Vergleich zur Kontrolle nach 5-tägiger Inkubation bei 32 °C auf. Mit *Lb. helveticus* I und M hergestellte Aufschwemmungen, die wegen des hohen enzymatischen Potentials und hoher autolytischer Eigenschaften ausgewählt waren, zeigten die höchsten Peptidase- und Esteraseaktivität, während Aufschwemmungen mit den schwach autolytischen Kulturen *Lb. casei* A und *Lb. helveticus* L geringe Enzymaktivität aufwiesen. Große Mengen an wasserlöslichem Stickstoff (WSN) und freien Aminosäuren (FAA) wurden in den Käseaufschwemmungen mit höchstem Zusatz im Vergleich zur Kontrolle ohne Zusatz festgestellt. *Lb. helveticus* I, M und U zeigten den stärksten Proteinabbau in den Aufschwemmungen – entsprechend den hohen WSN- und FAA-Gehalten als Folge der hohen Freisetzung an Peptidaseaktivität und geringer Resistenz gegen den autolytischen Prozeß. Die gegenüber der Autolyse sehr widerstandsfähigen *Lb. casei* A und *Lb. helveticus* L wiesen geringe Proteolyse nach 5 Tagen Reifung auf. Zusatz-behandelte Aufschwemmungen zeigten ausgeprägteren Proteinabbau bei der Polyacrylamid-Gelelektrophorese (PAGE) und bewiesen damit eine höhere Peptidaseaktivität im Bruch verglichen mit der Kontrolle. Die relative Konzentration von freien Fettsäuren (FFA) in Käsebruchaufschwemmungen belegte eine hohe Lipolyse in den meisten Zusatz-behandelten Aufschwemmungen. Eine höhere FFA-Menge wurde im Vergleich zu den Kontrollen in Aufschwemmungen mit Zusatz I, M, U und C freigesetzt. Eine Sensorikjury zeigte, daß bei Käsebruch-Aufschwemmungen mit Laktobazillen Käseflavour- und -aroma am besten bewertet wurden, die Flavourintensität jedoch bei den Aufschwemmungen mit unterschiedlichen Zusätzen variierte.

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